Journal of Insect Science, (2025) 25(3): 18; ieaf055
https://doi.org/10.1093/jisesa/ieaf055
Research

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Investigating a role for piRNA-associated *piwi* genes in overcoming host-plant resistance in the soybean aphid, *Aphis glycines*

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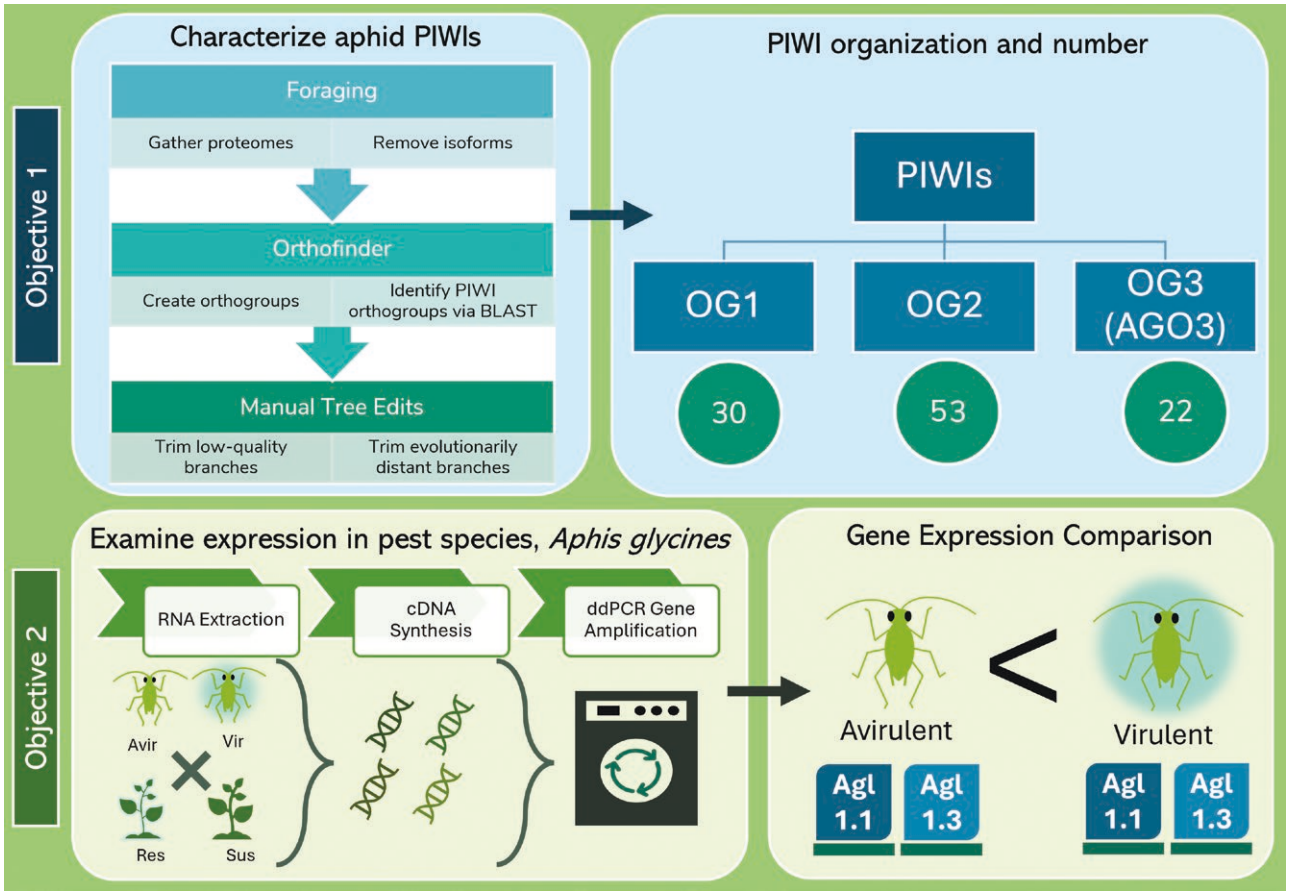
Subject Editor: Sheila Ons

Received on 5 August 2024; revised on 11 April 2025; accepted on 28 April 2025

Natural host-plant resistance provides a sustainable solution to control insect outbreaks but can be limited due to insect counter-adaptation. The exact mechanisms of insect adaptation to host-plant resistance remain unclear in most systems. Some insect adaptations are controlled by epigenetic mechanisms, such as through noncoding RNA. PIWI-interacting RNAs are specific noncoding RNAs that bind with PIWI proteins to control a diverse range of gene regulatory functions, particularly in insects. Previous investigation into aphid PIWI gene copies showed expansion in their abundance compared to other insects, which may suggest PIWI genes have additional functions among aphids. We first characterized PIWI gene evolution through a phylogenetic analysis, then investigated the role of PIWIs by examining gene expression in the soybean aphid (*Aphis glycines*), a significant insect pest of soybean which has adapted to overcome aphid-resistance in host plants. Our data indicated the presence of three PIWI ortholog groups, as well as taxon-specific gene expansions, with gene copy numbers ranging from 3 to 17 across species. To evaluate a potential role of PIWIs in overcoming host-plant resistance, we measured their gene expression in *Ap. glycines* with (virulent) and without (avirulent) the ability to survive on aphid-resistant soybean. We found that virulent *Ap. glycines* have significantly higher expression of 2 PIWI genes (*Agl1.1* and *Agl1.3*) compared to the avirulent biotype. These data suggest that gene regulatory mechanisms related to the PIWI pathway, potentially including piRNAs, are important in aphid systems and may enable adaptation to host-plant resistance.

Keywords: PIWI, host-plant resistance, adaptation, Aphididae

Graphical Abstract



Introduction

Host-plant resistance (HPR) against insects can provide a sustainable alternative to pesticides by utilizing natural defensive genes within plants that repel or protect them from insect feeding or colonization (Sharma and Ortiz 2002, Stout 2014). However, many insects have adapted to overcome these natural plant defenses, complicating the utility of HPR. Adaptation to HPR typically follows a gene-for-gene model (Simon et al. 2015, Stuart 2015). Mutations can occur in specific insect genes that either interact with plant defense pathways, such as effector proteins, or detoxify plant secondary metabolites, such as cytochrome P450s (Smith and Clement 2012, Smith and Chuang 2014). Recent research on insect adaptation suggests that mechanisms related to gene regulation, such as epigenetics and noncoding RNAs (ncRNA), may also facilitate adaptation related to host feeding (Glastad et al. 2011, Srinivasan and Brisson 2012, Duncan et al. 2014, Grantham et al. 2015, Brevik et al. 2018, Chen et al. 2020, Crossley et al. 2022). These mechanisms have not been fully explored in HPR systems, especially in aphids which have a propensity to overcome plant defenses (Srinivasan and Brisson 2012, Yates and Michel 2018).

One important and functionally diverse type of ncRNA is a subclass of small RNAs called piRNAs (PIWI-interacting RNAs), which are ~24 to 30 nt long and are generated from various heterochromatic regions called piRNA clusters. These clusters are hotspots for rapid evolution and often contain active or relic transposable elements (TEs) (Thomson and Lin 2009, Santos et al. 2023). TE regulation is perhaps the most well-known and important function of piRNAs since TEs can disrupt and damage genomes by interfering with gene structure and the expression of neighboring genes (Luo and Lu 2017, Santos et al. 2023). TE regulation is facilitated by a protein complex consisting of binding factors, piRNA, and an Argonaute subfamily of proteins known as PIWIs. The PIWI–piRNA complex uses the sequence information encoded in piRNAs to recognize and bind to gene targets. Along with TEs, PIWI–piRNA targets include certain chromatin regions and specific mRNAs that are post-transcriptionally silenced (Santos et al. 2023). While PIWIs have traditionally been thought to only be expressed in germline tissue, there is substantial evidence that they also play a role in insect somatic tissues (Thomson and Lin 2009, Lu et al. 2011, Ramat and Simonelig 2021). Most of our understanding of PIWI functions originate from three model insect species: *Drosophila melanogaster*, *Aedes aegypti*, and *Bombyx mori*. PIWIs and piRNA have unique functional roles across insect taxa, such as regulating TEs, gene splicing, fertility, maintaining telomeres, aiding in sex determination and embryonic development, and providing antiviral defense (Santos et al. 2023). This wide functional diversity may also include adaptation to plant defense, especially in aphids where epigenetic regulation is common (Sattar et al. 2012, Srinivasan and Brisson 2012, Breeds et al. 2018, Chen et al. 2020, Yates-Stewart et al. 2020).

Previous studies in aphids have found differential expression of PIWIs and piRNAs. An expansion of *piwi* genes occurred in the pea aphid (*Acyrthosiphon pisum*) compared to other insects (Lu et al. 2011). These *piwis* were also differentially expressed among different reproductive morphs and life stages. Certain PIWIs were

found to be specialized for germline or somatic functions, indicating involvement with the production of different reproductive morphs (Lu et al. 2011, Srinivasan and Brisson 2012, Santos et al. 2023). In the cotton aphid (*Aphis gossypii*), small RNA sequencing revealed that aphids had higher expression of piRNA-like elements when exposed to aphid-resistant plants than when exposed to susceptible plants (Sattar et al. 2012). Additional research in other aphid systems is important to gain a better understanding of the role of piRNAs and PIWIs for aphid–plant interactions.

Here we characterized PIWIs in the soybean aphid, *Aphis glycines*, specifically in relation to overcoming HPR. This aphid is an economically important and invasive pest across North America, significantly reducing soybean yield (Kim et al. 2008). To manage *Ap. glycines* in fields, growers have increased insecticide applications by 130% in less than 10 yr after its introduction (Hodgson et al. 2012). As an alternative and more sustainable solution, HPR genes have been explored as a preventative measure against aphid infestations (Bansal et al. 2014, Natukunda et al. 2021). HPR against *Ap. glycines* through *Rag* (Resistance to *Ap. glycines*) genes provides control of these pests without the need for pesticides (Hesler et al. 2013, Ajayi-Oyetunde et al. 2016). However, the utility of resistant soybean varieties may be limited by the emergence of virulent aphid biotypes that can overcome HPR (Tilmon et al. 2021). Although the exact mechanisms of virulence evolution are unknown, previous research suggests that virulent (biotype 4, or B4) aphids have higher gene expression of several TE families (Yates-Stewart et al. 2020). Differential TE expression could be influenced by gene regulatory mechanisms such as the PIWI pathway (Thomson and Lin 2009, Luo and Lu 2017, Santos et al. 2023). In this study, we characterized the evolution of known and predicted PIWI proteins across aphids with available annotated proteomes, including *Ap. glycines*. Then, we compared *piwi* gene expression between avirulent (biotype 1, or B1) and virulent (B4) *Ap. glycines* aphids to determine if expression is associated with virulence. Our data indicates potential new functional roles for *piwi* genes in aphid–plant interactions, including virulence adaptation.

Materials and Methods

Phylogenetic Trees

All available Aphididea proteomes ($n = 15$) were collected across NCBI Refseq/GenBank and AphidBase (www.aphidbase.com). If multiple proteomes were available, the selection preference was (i) the NCBI reference proteome, or (ii) the most recently updated version of the proteome. For proteins that produced multiple isoforms, only the longest isoform was kept for analysis. All phylogenetic analyses were performed using the Ohio Supercomputer (Ohio Supercomputer Center. 1987). Orthofinder v2.5.5 (Emms and Kelly 2019) was used to infer orthogroups among the complete proteomes. Orthofinder also runs FastTree v2.1.11 to construct phylogenetic trees for each orthogroup, and generates a species tree using all orthogroups that are present in all species with STAG (Emms and Kelly 2018). PIWI/Aub/AGO3 homologs were identified by searching the orthogroups for previously annotated PIWI genes in *Ac. pisum* (Lu et al. 2011), with relevant orthogroup trees retained for analysis. Protein accession numbers were manually searched on NCBI to confirm annotation as PIWI/Aub/AGO3 or the closely related SIWI. Proteins with different annotations were removed, while sequences that NCBI labeled as unidentified or of low-quality were marked. NCBI defines low quality predicted proteins as those that must be altered from their actual transcript to encode a functioning protein. This can be caused by errors in sequencing resulting in mismatches and/or frameshift mutations (Pruitt et al. 2020). If the longest branch was twice as long as the second longest branch, that protein was declared evolutionarily distant and trimmed from the final tree. Unaltered trees can be viewed in Supplementary Fig. 1A and 1B. Each PIWI-related orthogroup was analyzed separately with their own phylogenetic tree, then together within a single overall tree (Supplementary Fig. 2). Proteins (and genes encoding them) were each given a unique, simplified label independent of accession number (see Supplementary Table 1 for all accession numbers). Names included an abbreviation of the aphid species, the orthogroup they were placed in, followed by sequential numbering. Branch lengths were calculated by Orthofinder by first performing an all-vs-all BLAST analysis, then standardizing the BLAST bit scores to eliminate length bias. These normalized scores were used to infer orthogroups, which were subsequently clustered using a Markov Clustering algorithm with statistical support via bootstrap analysis. Final trees were built in R v.4.4.1 with ggtree v.1.14.6 (Yu et al. 2017). To create the single overall tree, unaligned sequences for the three focal orthogroups were collected from the "Orthogroup_Sequences" folder within the Orthofinder output, concatenated into a single FASTA file, and aligned with MAFFT v. 7.520 (Katoh and Standley 2013) using default parameters. The aligned sequences were trimmed with ClipKIT v. 2.3.0 (Steenwyk et al. 2020) using default parameters and subjected to phylogenetic inference with IQ-Tree v. 2.2.2.7 (Minh et al. 2020) using 1,000 ultrafast bootstraps (Hoang et al. 2018) and otherwise with default parameters.

DNA and Amino Acid Sequence Alignments

To assess the functionality of PIWI proteins found in *Ap. glycines*, an amino acid sequence alignment was inspected for the presence of critical sites and domains (Lu et al. 2011) (see Supplementary Document 1). To check for the presence of unique B4 alleles, a protein vs protein BLAST search was performed on AphidBase hosted at the Bioinformatics Platform for Agroecosystem Arthropods (BIPA: https://bipaa.genouest.org/is/aphidbase/) using the B1 PIWI sequences against the B4 transcriptome (*Ap. glycines* annotation v1.0 Ag b4 proteins) (Altschul et al. 1997) . If differences were observed, each allele was added to the alignment to assess functional differences. The alignment was performed in Geneious Prime using a Clustal algorithm. A representative sequence from each orthogroup found in *Ac. pisum* was included to validate critical sections. A nucleotide alignment was also generated. Coding sequences were found previously by BLASTing the protein sequences against the *Ap. glycines* coding sequence database (*Ap. glycines* annotation v6.0 Ag bt1 CDS) within AphidBase. The coding sequence for Agl1.2, which did not appear in this search, was found by searching the protein accession number acquired by Orthofinder on NCBI, then accessing the linked nucleotide sequence. The alignment was also created in Geneious Prime using the Clustal algorithm with default parameters.

Gene Expression

Ap. glycines Strains and Rearing

Experiments were performed on *Ap. glycines* biotypes 1 (B1, avirulent) and 4 (B4, virulent). These colonies have been maintained in the Michel laboratory for several years, derived from an *Ap. glycines* stock center at the University of Illinois. Aphids were reared in growth chambers set to a high of 27 °C and a 14:10 h photoperiod (Bansal et al. 2014). B1 colonies are reared on aphid susceptible soybean containing no aphid-resistant *Rag* genes (LD14-8007) while

B4 are reared on a resistant soybean variety that contains both *Rag1* and *Rag2* genes (LD14-8001).

Experimental Design

Aphids were age synchronized 1 wk prior to experiments and maintained on a detached leaf placed within a Parafilm sealed, charcoal-lined petri dish (Li et al. 2004). Aphids were starved to promote feeding prior to experimental start by transferring them to a new Petri dish without detached leaves for 24 h. One-wk-old adult aphids of both biotypes were placed on 2-wk-old aphid susceptible and *Rag1*/*Rag2* soybean. Each biological replicate had all 4 combinations of biotype and plant (B1 on susceptible and *Rag1*/*Rag2*; B4 on susceptible and *Rag1*/*Rag2*). Each plant had 10 aphids, distributed equally within a custom 3D printed clip cage (5 aphids per cage, see Supplementary Fig. 3A). Six total biological replicates were collected, with replicates 1-3 collected separately from 4 to 6. Aphids fed for 24 h and then were collected using a specially designed 3D-printed aspirator (Supplementary Fig. 3B), placing aphids directly into 1.5 ml microcentrifuge tubes. 3D printing parameters are available upon request. Aphids were immediately flash frozen by placing tubes in liquid nitrogen, then stored at −80°C.

Sample Preparation

RNA was extracted from 3 or 5 pooled aphids (3 aphids for replicates 1-3, 5 aphids for replicates 4-6) using Qiagen's RNeasy mini kit following the manufacturer's protocol. To ensure RNA purity, the optional steps of DNAse digestion and final drying were performed. For a more concentrated sample, 30 µl of RNAse free water was added to the column and samples were incubated for 15 min at room temperature before elution. Sample concentrations were standardized within each replicate using a Nanodrop 2000 (Thermo, USA). cDNA was generated using BioRad's iScript cDNA Synthesis Kit following the manufacturer's protocol. Concentrations were standardized again to 100 ng/µl using a Nanodrop 2000, then stored at −20°C.

Gene Expression Analysis

The genes for expression analysis included 7 *Ap. glycines piwi* genes (including 1 *ago3* gene), herein shortened as: *Agl1.1* (*KAE9523124*), *Agl1.2* (*KAE9532669*), *Agl1.3* (*KAE9524190*), *Agl1.4* (*KAE9524184*), *Agl1.5* (*KAE9523825*), *Agl2.6* (*KAE9525450*), and *Agl.AGO3.1* (*KAE9533345*). The reference gene *RPS9* encodes a ribosomal protein, which is a stable reference gene for experiments involving soybean aphids feeding on both resistant and susceptible plants (Bansal et al. 2012). Primers for all PIWIs except *Agl1.2* were designed by NCBI's primer design tool using the coding sequence of each gene. All suggested primers were examined in a Clustal multiple sequence alignment performed in Geneious Prime to confirm minimal primer sequence overlap with off-target *piwi* genes. Primers with at least 2 mismatches compared to other PIWI sequences were selected (see Supplementary Document 2). *Agl1.1* is the only exception, with the forward primer matching a sequence in *Agl1.2*; however Sanger sequencing confirmed primer specificity (data not shown). Primers for *Agl1.2* were designed by hand within Geneious Prime to span a unique deletion and ensure specificity, as NCBI's primers resulted in nonspecific amplification. All primer and semiquantitative PCR conditions can be found in Supplementary Table 2. Gene expression was first evaluated with semiquantitative PCR using a cDNA template and examined on a 2% agarose gel. Primers for *Agl1.2* were functionally validated by using genomic DNA templates in a PCR reaction. Band intensity of expressed genes was measured via ImageJ using three replicates (data summarized in Supplementary Table 3), and an ANOVA was performed to detect any preliminary differential expression patterns (Supplementary Document 3). Genes that had a significant difference in band intensity between biotypes ($P < 0.05$), or a presence/absence pattern were selected for droplet digital PCR (ddPCR) analysis. Due to the limited sensitivity of semiquantitative PCR, we also included 1 gene without significant differences in band intensity (*Agl2.6*) in our ddPCR analysis to validate ImageJ results.

In brief, ddPCR measures absolute gene expression levels through the generation of thousands of droplets, each containing materials for individual PCR, including fluorescence. After PCR, the number of droplets with fluorescence (ie a positive result) for both a gene of interest and reference gene are counted. A normalized gene expression ratio value is calculated, which can then be compared across samples and treatments. The program employs Poisson statistics to provide an absolute quantification of target DNA (Begum et al. 2021, Mavridis et al. 2022). Protocols for ddPCR followed standard sample preparation according to BioRad's instructions. The optimized concentrations and conditions for all *piwi* genes evaluated can be found in Supplementary Table 4. A total 20 µl of the sample mix was placed into a BioRad DG8 cartridge along with 70 µl of droplet generation oil. The cartridge was placed into BioRad's QX200 droplet generator which created the necessary droplets. The resulting mixtures (40 µl) were carefully transferred onto a 96-well plate. Plates were heat sealed using BioRad's PX1PCR plate sealer, then transferred to a C1000Touch Thermal Cycler Deep Well Reaction Module (BioRad) for PCR. Positive and negative droplets were counted on the QX200 Droplet Reader. Concentration (copies/µl) was measured using QX Manager 2.0 Standard Edition (BioRad). Positive droplet thresholds were manually inspected and adjusted, if needed, following the program's automatic estimations to ensure accuracy.

Statistical Analysis

To measure and compare gene expression, the ratio value of each sample was calculated as: [gene of interest]/[reference gene]. A Shapiro–Wilk test was used to check for sample normality within biotypes. Data transformations were performed if necessary to pass this normality test: *Agl1.3* underwent a log transformation calculated by the log function in R v4.4.1, while *Agl1.4* and *Agl1.1* underwent a square root transformation calculated by the sqrt function in R v4.4.1. Data collected from replicates 1-3 showed no significant difference from replicates 4-6, thus all replicate data were pooled for analysis. An ANOVA was performed for each gene using a linear model (R package nlme v3.1-164) to detect any interactions. Significance was defined as having a P value < 0.05. After determining that neither plant treatment (ie susceptible or resistant) had any effect on gene expression through the ANOVA and as supported by previous studies (Yates-Stewart et al. 2020), plant treatments were pooled when comparing significant differences in gene expression among biotypes. Figures were created with all raw, untransformed data pooled within biotypes using ggplot2 v3.4.4, and ggpubr v0.6.0 in R v4.4.1.

Results

PIWI Phylogenetics Across Aphididae

Species Tree

For comparison with previous aphid phylogenies, a species tree was created using Orthofinder based on all 19,075 orthogroups that occurred in all included species (Fig. 1). As expected, all 15

included aphid species were grouped accordingly by subfamily, with monophyletic relationships for the main tribes of Aphidini (6 species) and Macrosiphini (6 species). Outgroups to the Aphidinae family included 3 species: *Sipha flava*, *Eriosoma lanigerum*, and *Cinara cedri*.

PIWI Orthogroups and Phylogenetic Trees

We found three PIWI orthogroups (OGs) among the available aphid proteomes: Orthogroup 1 (OG1) and 2 (OG2) contained PIWIs whereas OG3 included the AGO3s, aligning with previous data (Lu et al. 2011). One protein was too distantly related in OG1 and was trimmed (Supplementary Fig 1A), 2 were trimmed in OG2 (Supplementary Fig 1B), while none were trimmed in the AGO3 group. The number of PIWI proteins varied greatly across the 15 Aphididae members, with a total of 105 proteins identified, ranging from 3 to 17 proteins in a species (Table 1). Despite this, all species had at least 1 AGO3 protein and 1 protein in OG2; we did not find proteins in OG1 for *Metopolophium dirhodum*, *Melanaphis sacchari*, or *C. cedri*. All trees from the three OGs further supported the monophyletic relationship of the Aphidinae subfamily, with *S. flava*, *C.*

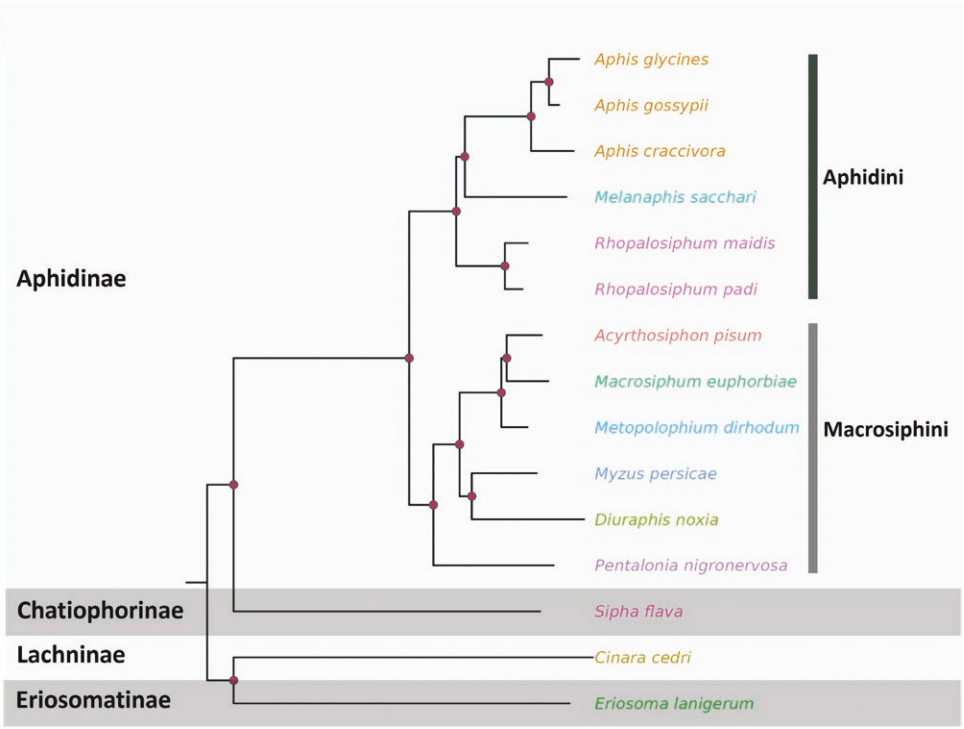


Fig. 1. Overall phylogenetic tree of certain aphid species within Aphididae. All available aphid proteomes were pulled from Refseq, Genbank, and Aphidbase to supply Orthofinder. An overall predicted phylogenetic species tree was generated based on all orthologous groupings. All nodes had a bootstrap support of 1, which was calculated with a Markov Clustering algorithm performed by OrthoMCL as part of the Orthofinder pipeline (Li et al. 2003, Emms and Kelly 2015).

Table 1. Summary of PIWI orthologous groupings across 15 Aphididea lineages

Species	Subfamily	Tribe	Subtribe	OG1[a] PIWI	OG2[b] PIWI	OG3 AGO3
Acyrthosiphon pisum	Aphidinea	Macrosiphini		5	7	5
Myzus persicae	Aphidinea	Macrosiphini		1	4	2
Macrosiphum euphorbiae	Aphidinea	Macrosiphini		2	7	1
Diuraphis noxia	Aphidinea	Macrosiphini		2	2	1
Metopolophium dirhodum	Aphidinea	Macrosiphini		0	5	2
Pentalonia nigronervosa	Aphidinea	Macrosiphini		4	3	1
Aphis gossypii	Aphidinea	Aphidini	Aphidina	4	1	1
Aphis glycines	Aphidinea	Aphidini	Aphidina	5	1	1
Aphis craccivora	Aphidinea	Aphidini	Aphidina	3	1	1
Rhopalosiphum padi	Aphidinea	Aphidini	Rhopalosiphina	1	6	1
Rhopalosiphum maidis	Aphidinea	Aphidini	Rhopalosiphina	1	3	1
Melanaphis sacchari	Aphidinea	Aphidini	Rhopalosiphina	0	8	1
Cinara cedri	Lachninae	Eulachnini		0	3	1
Sipha flava	Chatiophorinae			1	1	2
Eriosoma lanigerum	Eriosomatinae	Eriosomatini		1	1	1

Abbreviations: OG = orthogroup.

[a]One protein was excluded (Api1.4).

[b]Two proteins were excluded (Api2.7, Cce2.4).

cedri, and *E. lanigerum* consistently serving as outgroups. However, some PIWIs in OG1 and OG2 did not cluster monophyletically, with PIWIs from some species clustering with aphids from different subtribes (Fig. 2). Overall, the two main tribes in our analysis (Aphidini and Macrosiphini) differed in the numbers of PIWIs in each OG. For OG1, both tribes had 14 proteins across 6 species. Conversely, for OG2, Macrosiphini had 28 proteins and Aphidini 20 proteins (Table 1). When all three orthogroups were mapped together, OG3 clustered monophyletically. Two proteins from OG2 grouped with OG1, however these same two proteins were observed to be outgroups in the OG2 only tree, confirming their evolutionary distance.

OG1 was characterized by a large variation of PIWI proteins per species independent of tribe and subtribe (30 total genes). *Ac. pisum* (Macrosiphini) and *Ap. glycines* (Aphidini) had the highest number of PIWIs (5), followed by 4 PIWIs in *Pentalonia nigronervosa* (Macrosiphini) and *Ap. gossypii* (Aphidini), and 3 PIWIs in *Aphis craccivora* (Aphidini) (Table 1). In fact, species in the *Aphis* genus were overrepresented in OG1 compared to other Aphidini genera with *Rhopolasiphum* species only having 1 PIWI, and *Melanaphis* with none. In general, the OG1 protein tree reflected known Aphididae phylogeny, except for 2 *Ac. pisum* PIWIs (Api1.5 and Api1.6) clustering with *P. nigronervosa* (Fig. 2A).

OG2 had the highest total number of PIWIs (53), almost twice as many as OG1. All Macrosiphini species had more than 2 PIWIs, ranging from 2 (*Diuraphis noxia*) to 7 (*Ac. pisum* and *Macrosiphum euphorbiae*, Fig. 2B). This expansion occurred in only two Aphidini genera, *Rhopalasiphum* and *Melanaphis*. Surprisingly, all three *Aphis* species only had 1 PIWI in OG2, despite the expansion in OG1. All OG2 PIWIs grouped in two clusters, reflecting a similar pattern found in *Ac. pisum* (Lu et al. 2011). The first cluster did not contain any PIWIs from *Aphis*, but the remaining Aphidini formed a polyphyletic group (which also included 1 sequence from *Ac. pisum* and 2 from *P. nigronervosa*). The second group in this cluster contained PIWIs from Macrosiphini, and was polyphyletic, with evidence of species-specific expansion as seen with *Myzus persicae* and *Ma. euphorbiae*. The second OG2 cluster had mostly monophyletic grouping by tribes instead of by PIWI. In this cluster, the Aphidini had PIWIs from all three genera, including those from *Aphis*, although *Melanaphis* was an outgroup to *Aphis* and *Rhopalosiphum*. The Macrosiphini were also monophyletic, with expansions in *Ac. pisum*, *Ma. euphorbiae*, and *Met. dirhodum*.

OG3 represented the AGO3 proteins, and had the lowest total number, with only a few species having multiple copies (Fig. 2C). Many of these had long branches such as Sfla.AGO3.2 and Api. AGO3.5 which might suggest reduced or modified protein functionality. Monophyly was mostly seen with OG3, except for 2 AGO3s from *Ac. pisum* (Api.AGO3.4 and Api.AGO3.5), and a predicted duplicate from *My. persicae*. Like in other trees, *Me. sacchari* was the outgroup to *Aphis* and *Rhopolasiphum*, instead of the typical clustering of *Aphis* with *Melanaphis* as seen in the species tree.

Functional Characterization of *Ap. glycines* PIWI Sequences

Prior to measuring gene expression, we investigated the 6 PIWI and 1 AGO3 sequences in *Ap. glycines* for the presence of functional regions such as the PAZ, MID, and PIWI domains, as well as critical sites within each domain. The *Ap. glycines* AGO3 is 930 amino acids long, which is 162 less than *Ac. pisum*'s AGO3.4 (Api.AGO3.4's sequence was chosen for the sequence alignment as it has been previously identified [Lu et al. 2011] and has a well conserved sequence). The *Ap. glycines* AGO3 contained all critical residues and domains, consistent with the conservation seen in the OG3 tree. Most other *Ap. glycines* PIWIs showed conservation at important sites. The main exception was Agl1.2, which is likely not functional as a PIWI since it is missing 162 amino acids that contain four important functional residues across the MID and PIWI domains (Supplementary Document 1). This is corroborated by the fact *Agl1.2*-specific primers only showed amplification on gDNA templates, and none on cDNA, indicating that it is not expressed. Notably, Agl1.1 is 18 residues shorter at the C-terminus compared to all other PIWIs. Further investigation with the B1 cds database (via Aphidbase) revealed 2 alleles for this protein depending on the biotype. The B1 and B4 alleles have 84.8% sequence similarity, with the 18 residue deletion unique to the B1 allele. Despite sequence differences, both alleles have all the critical PIWI residues. Similar to Agl1.1, Agl1.5 also had 93 amino acids missing at the C-terminus of the sequence, but this deletion contained one key residue (histidine). Interestingly, this deletion is also unique to the B1 allele, with the B4 sequence possessing a consistent length with all other PIWIs. Overall, the two alleles had a 69.3% sequence similarity. The third PIWI to exhibit biotype allelic differences was Agl1.4, wherein the B4 allele had 7 less amino acids compared to the B1 allele toward the N-terminus, however this region contains no critical residues. All other PIWIs contained the critical domains and functional residues characteristic of PIWI proteins (Supplementary Document 1).

piwi Gene Expression

Since PIWI proteins can be involved in regulating TE expression, we compared their gene expression among *Ap. glycines* biotypes as a potential explanation for certain TE overexpression in B4 aphids (Yates-Stewart et al. 2020). We initially compared the expression of the 7 *piwi* (including AGO3) genes between biotypes fed different soybean plants by semiquantitative PCR. All genes except *Agl1.2* were expressed, yet, *Agl1.5*, *Agl2.6*, and *Agl.AGO3.1* showed no significant difference in expression via an Image J analysis (Supplementary Document). The remaining 3 PIWI genes (*Agl1.1*, *Agl1.3*, and *Agl1.4*) were further analyzed with ddPCR, along with *Agl2.6*, to confirm semiquantitative PCR results. We did not see significant differences among replicates, nor among plant types across any of the *piwi* genes ($P > 0.6$ for all genes, Supplementary Table 5). PIWIs *Agl1.4* and *Agl2.6* did not significantly differ in expression among biotypes ($P < 0.1$, Fig. 3). However, *Agl1.1* and *Agl1.3* had significantly higher gene expression in B4 aphids by a factor of 70.1 ($P > 0.001$) and 7.0 ($P < 0.001$), respectively (Fig. 3). Indeed, all replicates of B1 aphids consistently had almost no expression of *Agl1.1*.

Discussion

The success of HPR depends on the development of strategies that extend its durability and limit virulence adaptation. Thus, it is important to investigate all mechanisms that could overcome HPR that may be outside traditional gene-for-gene models (Smith and Chuang 2014, Simon et al. 2015, Stuart 2015). Recent research suggests that ncRNAs and other epigenetic pathways may be involved in insect adaptation, such as the functionally diverse PIWI/piRNA pathway (Sattar et al. 2012, Breeds et al. 2018, Wang and Lin 2021). We found a large quantity of PIWI genes across 15 aphid species with diverse evolutionary trajectories and taxon-specific expansion. We also found differential gene expression of two *piwi* genes between virulent and avirulent *Ap. glycines* biotypes. Understanding the

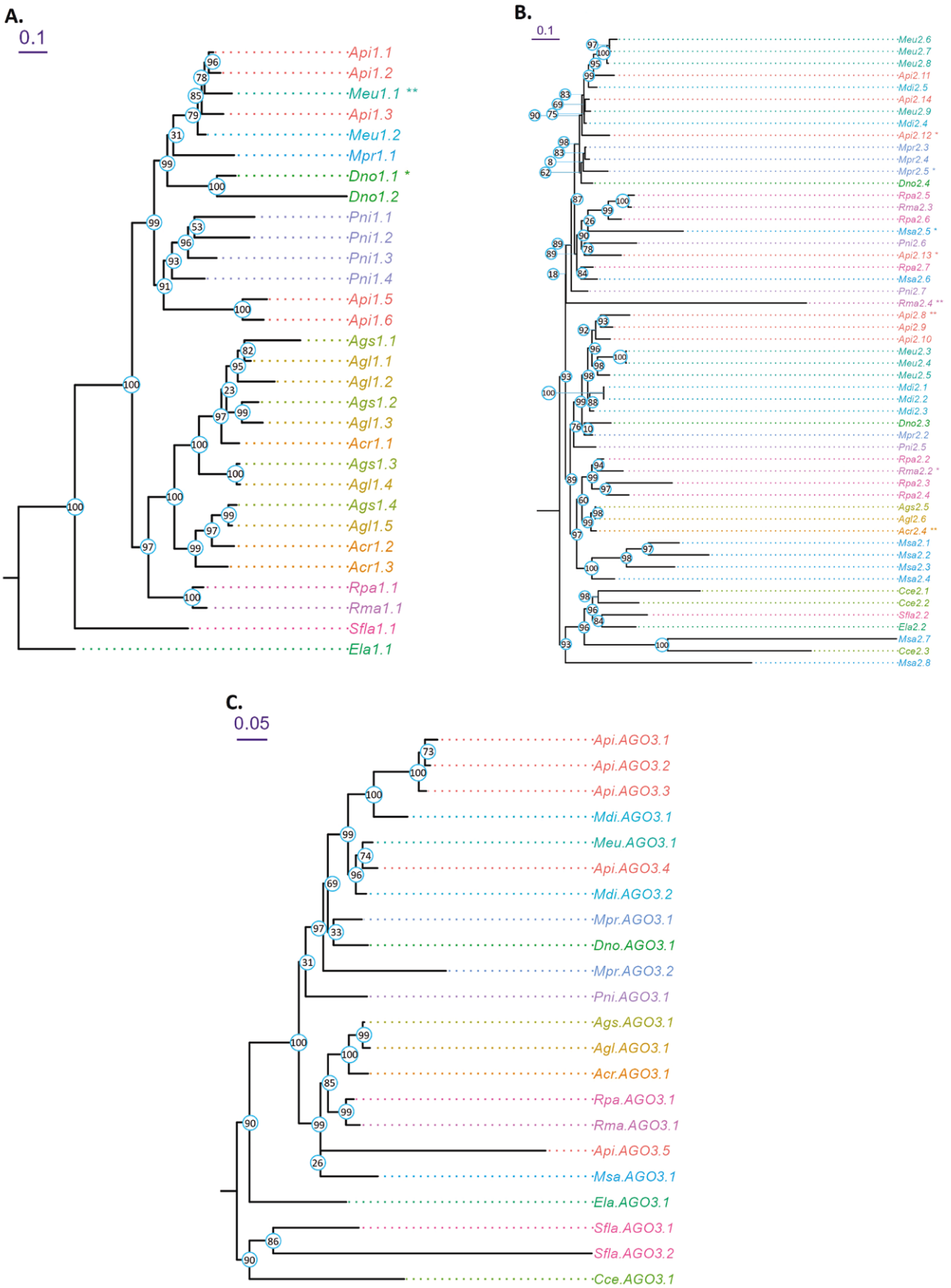


Fig. 2. Phylogenetic trees showing evolution of PIWI proteins within Aphididea. Phylogenetic tree data was generated by Orthofinder. Known PIWI genes (Lu et al. 2011) were used to identify potential PIWI orthologues across 15 aphid species within the family Aphididea: *Acyrthosiphon pisum (Api), Macrosiphum euphorbiae (Meu), Myzus persicae (Mpr), Diuraphis noxia (Dno), Pentalonia nigronervosa (Pni), Aphis gossypii (Ags), Aphis glycines (Agl), Aphis craccivora (Acr), Rhopalosiphum padi (Rpa), Rhopalosiphum maidis (Rma), Sipha flava (Sfla), Eriosoma lanigerum (Ela), Melanaphis sacchari (Msa), Metopolophium dirhodum (Mdi), Cinara cedri (Cce).* All known PIWIs sorted into three orthogroups, which were plotted in a phylogenetic tree. Orthogroup 1 (A) and orthogroup 2 (B) are potential PIWIs, while orthogroup 3 (C) contains potential AGO3s. Bootstrap support was calculated by Orthofinder through a Markov Clustering algorithm. Certain proteins are marked as unidentified (*) or low quality (**) as determined by NCBI.

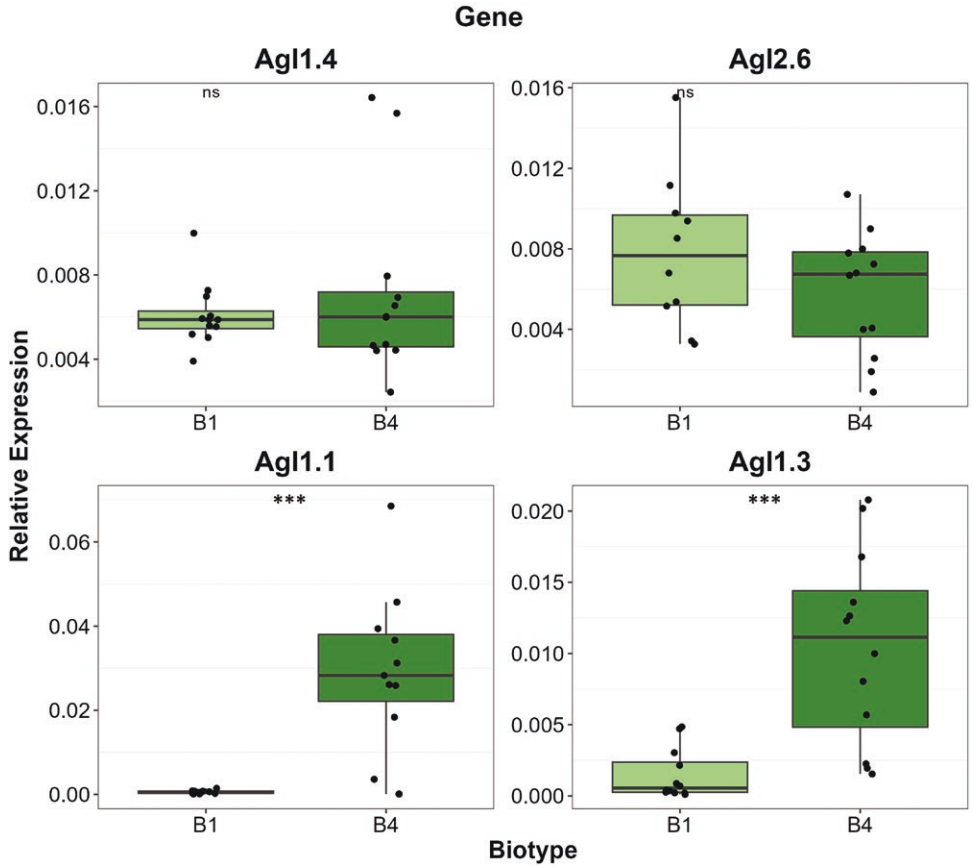


Fig. 3. Comparison of *piwi* gene expression between two *Ap. glycines* biotypes. ddPCR was used to measure the gene expression levels of 3 to 5 pooled adult aphids per treatment after feeding on soybean for 24 h. Relative expression was calculated by standardizing expression of each target gene to a reference (*RPS9,* a ribosomal gene). An ANOVA was performed for each gene, which determined that plant type had no effect, thus data were pooled by biotype. *Agl1.1* was expressed 70.1 times higher in virulent (B4) aphids ($P < 0.001$). *Agl1.3* had a similar pattern, with virulent aphids expressing it 7 times higher ($P < 0.001$). The other 2 genes had very similar levels of expression between biotypes ($P > 0.1$).

evolutionary implications of these proteins and uncovering their functions can lead to deeper insights of their role in insect-plant interactions and virulence adaptation.

Complex Evolutionary History and Expansion of Aphididae PIWIs

PIWI proteins are functionally diverse and associated with rapidly evolving ncRNAs (Santos et al. 2023); therefore examining the relationship of PIWIs across aphid taxa could increase our understanding of their importance in aphid life histories. Using 15 available aphid proteomes, aphid PIWIs were sorted into three orthogroups, consistent with earlier research focused on *Ac. pisum* (Lu et al. 2011). The number of potential *piwi* genes per aphid species is among the highest across insects currently seen, ranging from 3 (*C. cedri*) to 17 (*Ac. pisum*). Although studies from other insect groups are limited, mosquitoes (Culicidae) have a smaller range across species with 1 PIWI (AGO3) in *Anopheles darlingi* and 7 PIWIs in *Aedes aegypti* (Lu et al. 2011, Lewis et al. 2016). This difference among insect taxa could reflect unique or specialized roles for PIWI proteins. For example, *Drosophila* PIWIs are heavily involved in gametic cell line expression and regulation of TEs (Cd et al. 2009, Ramat and Simonelig 2021, Santos et al. 2023). While evidence suggests similar functions in other insects, PIWIs also play a unique and important role in defense against viruses that infect mosquitoes (Lewis et al. 2016, Santos et al. 2023). On the other hand, differences in the number of *piwi* genes could also be a result of inconsistent genomic resources and/or their quality. Whereas some insects and groups have well represented and annotated genomes, the complexity and genome sizes of other groups (such as Coleoptera and Orthoptera) make extrapolations and comparisons more challenging. Nonetheless, our evidence suggests that expansion and diversification first found in *Ac. pisum* (Lu et al. 2011) is present across much of the Aphididae family.

Our analysis showed substantial complexity in aphid PIWI evolution. An overall tree containing all PIWI proteins corroborates the distinction of three diverging orthogroups (Supplementaryl Fig. 2), with OG1 and OG2 having a closer relationship compared to OG3. Only three discrepancies emerged between the overall tree and the Orthofinder orthogroups, involving the proteins Ela1.1, Msa2.7, and Cce2.3. These discrepancies are likely due to the difficulties involved with accurately placing highly divergent sequences, since all three are either the most divergent sequence or have some of the longest branches within their orthogroup. Additionally, the Orthofinder algorithm for inferring orthogroups differs from the phylogenetic method implemented in IQTREE, which may also lead to different placements of individual proteins.

Among the three aphid orthogroups, OG3, which represented the AGO3s, showed the least difference in evolution pattern across species. All aphid species had at least 1 AGO3, and only four species had more than 2 copies (*Ac. pisum*, *My. persicae*, *Met. dirhodum*, and *S. flava*). An earlier study found 2 AGO3 proteins in *Ac. pisum*, with one likely being nonfunctional (Lu et al. 2011). Our approach identified 5 AGO3 proteins, with the original 2 Api.AGO3 proteins

grouping in different clusters, one with other Api.AGO3 proteins (Api.AGO3.3) and the other clustering between *Ma. euphorbiae* and *Met. dirhodum* proteins (Api.AGO3.4, Fig. 2C). This could be due to differences in our approach to identify PIWIs (ie Orthofinder) or access to a more recently updated proteome, although we did not include expression validation. *Ac. pisum* aside, there is relative conservation in the number of AGO3 proteins among aphid species. This pattern was not seen with OG1 and OG2. OG2 showed a similar topology as predicted with *Ac. pisum*, with two subclusters appearing after the split of Aphidinae from the aphid outgroups. Within tribes and genera, some PIWIs reflected monophyly, but most showed a complex evolutionary history. Many species had multiple copies distributed in both OG2 subgroups, with the exception of *Aphis*. The three *Aphis* species in our study only had 1 PIWI in OG2 without any subgrouping. Given that the closely related genera of *Rhopalospiphum* and *Melanaphis* had multiple OG2 genes similar to other species (Table 1), *Aphis* may have lost any duplications present in recent common ancestors, but this hypothesis remains to be tested.

Interestingly, OG1 had an alternative expansion pattern compared to OG2 within *Aphis*. *Aphis* was overrepresented with more copies in this orthogroup than the other two. This trend was unique to *Aphis* and the species *P. nigronervosa*. Overall, it may be that selection favored expansion and evolution in OG1 within *Aphis* rather than OG2. Additional data would be needed to fully evaluate this observation and identify potential selection pressures, such as sexual reproduction patterns (ie sexual, asexual, and mixed models) or host-feeding. While *piwi* genes are expressed across different reproductive life stages in *Ac. pisum*, similar data is lacking in other aphid species. This is especially true for species with obligatory sexual phases like *Ap. glycines*, which are difficult to generate in the laboratory. Additionally, differences in *piwi* duplications are likely not related to degrees of host specialization as *Ap. glycines* and its most closely related species *Ap. gossypii* have 5 and 4 PIWI proteins, respectively. Despite *Ap. glycines* being an extreme specialist (surviving only on soybean and its overwintering hosts within *Rhamnus*), *Ap. gossypii* is an extreme generalist that can feed on over 300 different plant species. As further research investigates evolutionary and possibly unique functional roles, more evidence may support the importance of OG1 PIWIs within *Aphis* that include interactions with their host plants.

Differences in *piwi* Gene Expression Suggest a Role in Virulence Adaptation

Differential expansion of *piwi* genes could provide evidence of new or specialized functional roles. To investigate the potential role of *piwi* genes contributing to *Ap. glycines* virulence, we compared their gene expression between adult virulent (B4) and avirulent (B1) aphids. Of the 7 total genes investigated, only 1 was not expressed by either biotype. While we attribute this to a loss-of-function deletion, it is possible that it could be expressed under different conditions or during different life stages. We identified two *Ap. glycines piwis* (*Agl1.1* and *Agl1.3*) that have significantly higher expression in virulent B4 aphids compared to avirulent B1 aphids (Fig. 3). This difference may be related to TE regulation, given previous evidence of PIWI function. However, previous studies found B4 aphids have overall higher expression of TE families, with only a few TEs experiencing comparative downregulation (Yates-Stewart et al. 2020). This is the opposite pattern to what we would expect—with an overall higher expression of TEs in B4, we should have observed less *piwi* expression due to increased regulation. It is possible that Agl1.1 and Agl1.3 are indeed causing the downregulation of some TE families observed in the same study, but any association with increased TE in virulent aphids remains unclear.

An alternative explanation may involve an increase in piRNAs in relation to HPR, similar to *Ap. gossypii* (Sattar et al. 2012). If piRNAs are associated with HPR feeding and virulence, then we may expect expression of specific PIWIs that are needed for the entire piRNA pathway. Additionally, it is possible the piRNAs involved in HPR target adaptive genes that must be differentially regulated using PIWI proteins as they do in other organisms (Wang and Lin 2021, Cai et al. 2022, Santos et al. 2023). Our future work will focus on comparing all small RNAs, including piRNAs, among B4 and B1 aphids to provide further evidence of their role in HPR.

We did identify three PIWIs with biotype-specific allele differences: *Agl1.1*, *Agl1.4*, and *Agl1.5*. Despite the differences, all critical residues remain present, except for the B1 allele of Agl1.5 which is missing one residue. These differences likely do not correlate with gene expression, since only one of these genes is differentially expressed. Interestingly, preliminary data from laboratory colonies suggest both biotypes are heterozygous at the *Agl1.1* locus (data not shown). In this study, we quantified expression on the RNA level and thus were unable to distinguish which allele was being expressed in B4 aphids. Even if both alleles/proteins were expressed, we are unable to determine if the allele with the premature stop codon may have a dominant effect (ie needing only 1 copy). Further, neither allele was expressed in B1 aphids, which had near-zero *Agl1.1* expression in our experiments. Yet, even with this premature stop and the overall 84.8% similarity between alleles, all the important residues are present in both (Supplementary Document 1) suggesting that any reduction in functionality of the truncated protein may be minimal. Nonetheless, all three of these polymorphisms will be further investigated as we next elucidate the highly specific piRNAs associated with these proteins.

Recent research has shown that the role of the piRNA pathway in insects has expanded from its original function of TE regulation in the germline (Ramat and Simonelig 2021, Santos et al. 2023). Here, we provide evidence of differential expansion and diversification of potential *piwi* genes across the Aphididae, with substantial variation among 15 aphid species. However, data from additional aphid species are needed to fully understand PIWI sequence evolution, including functional validation across different life stages as well as host plants. While our study supports the hypothesis that PIWIs and the piRNA pathway could contribute to aphid-plant interactions and overcoming plant resistance, an exact mechanism for this remains unclear. Through their interaction with PIWIs, piRNAs have many gene and pseudogene targets, interacting with other epigenetic mechanisms such as DNA methylation, histone modification, and lncRNA expression (Ramat and Simonelig 2021, Wang and Lin 2021, Santos et al. 2023). Future research may provide additional evidence for the importance and complexity of the role of PIWIs in aphids.

Acknowledgments

We would like to thank Erin Michel, Cindy Wallace and Ellie Lang for their help in maintaining our lab colonies and experimental support.

Author contributions

Angel Haller (Conceptualization [equal], Investigation [equal], Methodology [equal], Writing—original draft [equal], Writing—review & editing [equal]), Jelmer Poelstra (Conceptualization [supporting], Data curation [supporting], Formal analysis

[supporting], Methodology [supporting], Writing—review & editing [supporting]), Wirat Pipatpongpinyo (Data curation [supporting], Formal analysis [supporting], Methodology [supporting], Resources [supporting], Writing—review & editing [equal]), Nathan Kreuter (Conceptualization [equal], Formal analysis [supporting], Writing—review & editing [equal]), Jennifer Wilson (Formal analysis [equal], Investigation [equal], Methodology [equal], Writing—review & editing [equal]), and Andy Michel (Conceptualization [equal], Formal analysis [equal], Funding acquisition [lead], Investigation [equal], Methodology [equal], Writing—original draft [supporting], Writing—review & editing [equal])

Supplementary material

Supplementary material is available at *Journal of Insect Science* online.

Funding

This research was supported by The Ohio Soybean Council and the North Central Soybean Research Program.

Conflict of interest. The authors declare no conflict of interest.

Data availability

The data that support the findings of this study are available in the supplementary materials of this article.

References

Ajayi-Oyetunde OO, Diers BW, Lagos-Kutz D, et al. 2016. Differential reactions of soybean isolines with combinations of aphid resistance genes Rag1, Rag2, and Rag3 to four soybean aphid biotypes. J. Econ. Entomol. 109:1431–1437. https://doi.org/10.1093/jee/tow033

Altschul SF, Madden TL, Schäffer AA, et al. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 25:3389–3402.

Bansal R, Mamidala P, Mian MAR, et al. 2012. Validation of reference genes for gene expression studies in aphis glycines (Hemiptera: Aphididae). J. Econ. Entomol. 105:1432–1438. https://doi.org/10.1603/ec12095. [accessed 2024 Jan 10].

Bansal R, Mian M, Mittapalli O, Michel AP. 2014. RNA-Seq reveals a xenobiotic stress response in the soybean aphid, Aphis glycines, when fed aphid-resistant soybean. BMC Genomics. 15:972. https://doi.org/10.1186/1471-2164-15-972.

Begum M, Paul P, Das D, et al. 2021. Genes regulating development and behavior exhibited altered expression in Drosophila melanogaster exposed to bisphenol A: use of real-time quantitative PCR (qRT-PCR) and droplet digital PCR (ddPCR) in genotoxicity study. Environ. Sci. Pollut. Res. Int. 28:7090–7104. https://doi.org/10.1007/s11356-020-10805-0

Breeds K, Burger NFV, Botha A-M. 2018. New insights into the methylation status of virulent diuraphis noxia (Hemiptera: Aphididae) biotypes. J. Econ. Entomol. 111:1395–1403. https://doi.org/10.1093/jee/toy039.

Brevik K, Lindström L, McKay SD, Chen YH. 2018. Transgenerational effects of insecticides — implications for rapid pest evolution in agroecosystems. Curr. Opin. Insect Sci. 26:34–40. https://doi.org/10.1016/j.cois.2017.12.007.

Cai A, Hu Y, Zhou Z, et al. 2022. PIWI-interacting RNAs (piRNAs): promising applications as emerging biomarkers for digestive system cancer. Front. Mol. Biosci. 9. https://doi.org/10.3389/fmolb.2022.848105.

Chen Y, Singh A, Kaithakottil GG, et al. 2020. An aphid RNA transcript migrates systemically within plants and is a virulence factor. Proc. Natl. Acad. Sci. U.S.A. 117:12763–12771. https://doi.org/10.1073/pnas.1918410117.

Crossley MS, Cohen Z, Pélissié B, et al. 2022. Ecological and evolutionary factors mitigating Colorado potato beetle adaptation to insecticides. In: Alyokhin A, Rondon SI, Gao Y, editors. Insect pests of potato. 2nd ed. Academic Press. p. 463–479.

Duncan EJ, Gluckman PD, Dearden PK. 2014. Epigenetics, plasticity, and evolution: how do we link epigenetic change to phenotype? J. Exp. Zool Part B, Mol. Dev. Evol. 322:208–220. https://doi.org/10.1002/jez.b.22571.

Emms DM, Kelly S. 2015. OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. Genome Biol. 16(1):157. https://doi.org/10.1186/s13059-015-0721-2.

Emms DM, Kelly S. 2018. STAG: species tree inference from all genes.:267914. https://doi.org/10.1101/267914. https://www.biorxiv.org/content/10.1101/267914v1.

Emms DM, Kelly S. 2019. OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome Biol. 20:238. https://doi.org/10.1186/s13059-019-1832-y.

Glastad KM, Hunt BG, Yi SV, Goodisman MAD. 2011. DNA methylation in insects: on the brink of the epigenomic era. Insect Mol. Biol. 20:553–565. https://doi.org/10.1111/j.1365-2583.2011.01092.x

Grantham M, Brisson JA, Tagu D, Le Trionnaire G. 2015. Integrative genomic approaches to studying epigenetic mechanisms of phenotypic plasticity in the aphid. In: Raman C, Goldsmith MR, Agunbiade TA, editors. Short views on insect genomics and proteomics: insect genomics, Vol. 1. Springer International Publishing. p. 95–117. https://doi.org/10.1007/978-3-319-24235-4_5.

Hesler LS, Chiozza MV, O'Neal ME, et al. 2013. Performance and prospects of Rag genes for management of soybean aphid. Entomol. Exp. Appl. 147:201–216. https://doi.org/10.1111/eea.12073.

Hoang DT, Chernomor O, von Haeseler A, et al. 2018. UFBoot2: improving the ultrafast bootstrap approximation. Mol. Biol. Evol. 35:518–522. https://doi.org/10.1093/molbev/msx281.

Hodgson EW, McCornack BP, Tilmon K, Knodel JJ. 2012. Management recommendations for soybean aphid (Hemiptera: Aphididae) in the United States. J. Integr. Pest Manag. 3:E1–E10. https://doi.org/10.1603/ipm11019.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30:772–780. https://doi.org/10.1093/molbev/mst010

Kim CS, Schaible G, Garrett L, et al. 2008. Economic impacts of the U. S. soybean aphid infestation: a multi-regional competitive dynamic analysis. Agric. Resour. Econ. Rev. 37:227–242. https://doi.org/10.1017/s1068280500003026.

Lewis SH, Salmela H, Obbard DJ. 2016. Duplication and diversification of dipteran argonaute genes, and the evolutionary divergence of piwi and aubergine. Genome Biol. Evol. 8:507–518. https://doi.org/10.1093/gbe/evw018.

Li L, Stoeckert CJ, Roos DS. 2003. OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res. 13:2178–2189. https://doi.org/10.1101/gr.1224503.

Li Y, Hill CB, Hartman GL. 2004. Effect of three resistant soybean genotypes on the fecundity, mortality, and maturation of soybean aphid (Homoptera: Aphididae). J. Econ. Entomol. 97:1106–1111. https://doi.org/10.1093/jee/97.3.1106.

Lu H, Tanguy S, Rispe C, et al. 2011. Expansion of genes encoding piRNA-associated argonaute proteins in the pea aphid: diversification of expression profiles in different plastic morphs. PLOS One 6:e28051. https://doi.org/10.1371/journal.pone.0028051.

Luo S, Lu J. 2017. Silencing of transposable elements by piRNAs in drosophila: an evolutionary perspective. Genom Proteom. Bioinf. 15:164–176. https://doi.org/10.1016/j.gpb.2017.01.006.

Malone CD, Brennecke J, Dus M, et al. 2009. Specialized piRNA pathways act in germline and somatic tissues of the Drosophila ovary. Cell. 137(3). https://doi.org/10.1016/j.cell.2009.03.040.

Mavridis K, Papapostolou KM, Riga M, et al. 2022. Multiple TaqMan qPCR and droplet digital PCR (ddPCR) diagnostics for pesticide resistance monitoring and management, in the major agricultural pest Tetranychus urticae. Pest Manag. Sci. 78:263–273. https://doi.org/10.1002/ps.6632.

Minh BQ, Schmidt HA, Chernomor O, et al. 2020. IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol. Biol. Evol. 37:1530–1534. https://doi.org/10.1093/molbev/msaa015.

Natukunda MI, Hohenstein JD, McCabe CE, et al. 2021. Interaction between Rag genes results in a unique synergistic transcriptional response that enhances soybean resistance to soybean aphids. BMC Genomics. 22:887. https://doi.org/10.1186/s12864-021-08147-3.

Ohio Supercomputer Center. 1987. Ohio Supercomputer Center. http://osc.edu/ark:/19495/f5s1ph73.

Pruitt K, Murphy T, Brown G, Murphy M. 2020. RefSeq Frequently Asked Questions (FAQ). In: RefSeq Help [Internet]. National Center for Biotechnology Information (US). https://www.ncbi.nlm.nih.gov/books/NBK50679/ [accessed 24 January 2024].

Ramat A, Simonelig M. 2021. Functions of PIWI proteins in gene regulation: new arrows added to the piRNA quiver. Trends Genet. 37:188–200. https://doi.org/10.1016/j.tig.2020.08.011.

Santos D, Feng M, Kolliopoulou A, et al. 2023. What are the functional roles of piwi proteins and piRNAs in insects? Insects 14:187. https://doi.org/10.3390/insects14020187.

Sattar S, Addo-Quaye C, Song Y, et al. 2012. Expression of small RNA in aphis gossypii and its potential role in the resistance interaction with melon. PLOS One 7:e48579. https://doi.org/10.1371/journal.pone.0048579.

Sharma HC, Ortiz R. 2002. Host plant resistance to insects: an eco-friendly approach for pest management and environment conservation. J. Environ. Biol. 23:111–135.

Simon J-C, d'Alençon E, Guy E, et al. 2015. Genomics of adaptation to host-plants in herbivorous insects. Brief Funct. Genomics 14:413–423. https://doi.org/10.1093/bfgp/elv015.

Smith CM, Chuang W-P. 2014. Plant resistance to aphid feeding: behavioral, physiological, genetic and molecular cues regulate aphid host selection and feeding. Pest Manag. Sci. 70:528–540. https://doi.org/10.1002/ps.3689.

Smith CM, Clement SL. 2012. Molecular bases of plant resistance to arthropods. Annu Rev Entomol. 57:309–328. https://doi.org/10.1146/annurev-ento-120710-100642.

Srinivasan DG, Brisson JA. 2012. Aphids: a model for polyphenism and epigenetics. Genet Res Int. 2012:431531. https://doi.org/10.1155/2012/431531.

Steenwyk JL, Iii TJB, Li Y, et al. 2020. ClipKIT: a multiple sequence alignment trimming software for accurate phylogenomic inference. PLOS Biol. 18:e3001007. https://doi.org/10.1371/journal.pbio.3001007.

Stout MJ. 2014. Host-plant resistance in pest management. In: Abrol DP, editor. Integrated pest management. Academic Press. p. 1–21.

Stuart J. 2015. Insect effectors and gene-for-gene interactions with host plants. Curr. Opin. Insect Sci. 9:56–61. https://doi.org/10.1016/j.cois.2015.02.010.

Thomson T, Lin H. 2009. The biogenesis and function PIWI proteins and piRNAs: progress and prospect. Annu. Rev. Cell. Dev. Biol. 25:355–376. https://doi.org/10.1146/annurev.cellbio.24.110707.175327.

Tilmon KJ, Michel A, O'Neal ME. 2021. Aphid resistance is the future for soybean production, and has been since 2004: efforts towards a wider use of host plant resistance in soybean. Curr. Opin. Insect Sci. 45:53–58. https://doi.org/10.1016/j.cois.2021.01.003.

Wang C, Lin H. 2021. Roles of piRNAs in transposon and pseudogene regulation of germline mRNAs and lncRNAs. Genome Biol. 22:27. https://doi.org/10.1186/s13059-020-02221-x.

Yates AD, Michel A. 2018. Mechanisms of aphid adaptation to host plant resistance. Curr. Opin. Insect Sci. 26:41–49. https://doi.org/10.1016/j.cois.2018.01.003.

Yates-Stewart AD, Daron J, Wijeratne S, et al. 2020. Soybean aphids adapted to host-plant resistance by down regulating putative effectors and up regulating transposable elements. Insect Biochem. Mol. Biol. 121:103363. https://doi.org/10.1016/j.ibmb.2020.103363

Yu G, Smith DK, Zhu H, Guan Y, Lam TT-Y. 2017. ggtree: an r package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Methods Ecol. Evol. 8(1):28–36. https://doi.org/10.1111/2041-210x.12628.